JOURNAL OF SURGICAL RESEARCH 63, 355–358 (1996)
ARTICLE NO. 0275

Serial No.: 09/600,432
Group No.: 1653
Confirmation No.: 3387

Inhibition of Selectin- and Integrin-Mediated Inflammatory Response after Burn Injury

FIEMU E. NWARIAKU, M.D., PATRICIA J. SIKES, M.S., ELLIS LIGHTFOOT JR., AND WILLIAM J. MILESKI, M.D.

Department of Surgery, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9031

Presented at the Annual Meeting of the Association for Academic Surgery, Dearborn, Michigan, November 8–11, 1995

Inflammation and microvascular injury in the areas adjacent to burn wounds produces extension of postburn tissue necrosis. Leukocytes are potent mediators of th local inflammatory response preceding tissue necrosis, and the selectin and integrin adhesion molecules have been implicated in leukocyte-mediated tissue destruction. We sought to examine the role of L-selectin (CD62-L) and CD18 in leukocyte accumulation and tissue necrosis following burn injury. New Zealand White rabbits (n = 36) were subjected to burn injury and were randomized to treatment with saline (control) or monoclonal antibodies to L-selectin or CD18. Animals given the anti-L-selectin antibody demonstrated reduced immunohistochemical evidence of leukocyte accumulation at 24 hr postinjury but did not show improved wound perfusion or reduced tissue necrosis. Animals in the anti-CD18 group showed significantly improved tissue survival and improved tissue perfusion but had grades of leukocyte accumulation similar to those in the control group. These observations suggest that leukocyte accumulation is partially L-selectin dependent and that leukocyte accumulation alone is not sufficient to cause changes in blood flow and tissue destruction, both of which appear to be largely CD18 mediated.

INTRODUCTION

Thermal injury creates profound local and systemic derangements, both of which contribute to acute and chronic morbidity or death. At the local level thermal injury produces regions of irreversible tissue destruction surrounded by a marginal zone of injury with reduced blood flow [1–4]. In the postburn period, ongoing inflammation and microvascular injury in the zone of stasis results in progression of tissue loss which may require extensive surgical therapy by excision and skin grafting [5, 6]. Systemic morbidity is usually associated with larger burn size; this occurs frequently with wounds greater than 20% total body surface area.

Leukocytes, particularly polymorphonuclear neutrophils (PMN), are central mediators of injury in many acute pathologic processes. PMN-mediated injury is dependent in part on WBC adherence to the vascular endothelial cell (EC) surface. PMN–EC adherence results in the formation of a microenvironment between the PMN and the endothelial cell. In this microenvironment PMN-derived proteases and toxic oxygen products produced by both the endothelial cell and the PMN can exist in high local concentrations. These highly reactive substances, partially protected from inactivation by circulating plasma anti-proteases and free radical scavengers, may then produce endothelial cell injury resulting in intercellular gap formation, increases in microvascular permeability, edema, and thrombosis [7–11]. Investigation of the processes involved in leukocyte adherence have identified three main families of receptor/ligand molecules: integrins (CD11/CD18, VLA-4), selectins (E-selectin, P-selectin, L-selectin), and immunoglobulin supergene families (ICAM-1, VCAM-1) [12–23]. Specific monoclonal antibodies to the molecules involved in the leukocyte adherence process are under investigation as potential therapeutic agents for inflammatory conditions.

We tested the hypothesis that inhibition of leukocyte-endothelial adherence with the anti-CD18 monoclonal antibody R15.7 or the anti-L-selectin antibody Dreg 200 would reduce leukocyte accumulation and tissue necrosis following thermal injury.

METHODS

Local tissue injury. A model of thermal injury was developed using New Zealand White rabbits (1.8–2.3 kg). The animals' backs were shaved and venous access was obtained by cannulation of a peripheral ear vein with an angiocath (24 gauge). Cutaneous blood flow measurement was performed using a laser Doppler blood flow meter (PF 4000; Perimed, Inc., Piscataway, NJ) and an integrating flow probe (PF 313; Perimed, Inc.) containing 7 efferent laser fibers and 14 afferent fibers which reflect capillary perfusion in a tissue volume of approximately 1200 mm^3 (Perimed, Inc.). Previous studies have shown good correlation between laser Doppler blood flow measurement and standard radiolabeled microsphere calculations of blood flow [24–29]. Leukocyte counts and hematocrits were obtained at baseline, immediately postburn, and at 24, 48, and 72 hr postburn. Under general anesthesia (isoflurane) two sets of three full-thickness burns separated by two 5 × 30-mm zones were produced by applying brass probes heated to 100°C to the animals' backs for 30 sec. Baseline blood flow was measured at designated burned sites, marginal zones, and shaved unburned skin sites and was repeated 4, 24, 48,

0022-4804/96 $18.00

and 72 hr postburn. There were three experimental groups: control animals given saline alone, $n = 12$, and two groups of animals given anti-CD18 antibody (R15.7) and anti-L-selectin antibody (Dreg 200) at doses of 2.0 mg/ml. This dose results in circulating levels of antibody 10 μg/ml of plasma at 24 hr postinjection and was selected based on previously reported efficacy in the studies cited above. All animals were given analgesic (buprenorphine 0.05 mg/kg iv every 12 hr) throughout the study period. Animals in each group were anesthetized with ketamine (20 mg/kg iv) and biopsies of one burn site were obtained at 24 hr postburn, for histologic comparison of burn depth, edema, and leukocyte infiltration. At 72 hr postburn, the zones between the burn sites were evaluated for gross evidence of progression, and the number of zones with complete progression to confluent necrosis between burn sites was tabulated. Animals were euthanized at the conclusion of the 4-day study with a lethal intravenous injection of pentobarbital (150 mg/kg).

Leukocyte accumulation in the zone of stasis was performed by immunohistochemical evaluation of tissue staining for the leukocyte antigen CD11a using the monoclonal antibody R7.1. Tissue staining was graded on a scale of 0 to 4, with 0 = no staining, 1 = rare isolated staining, 2 = positive staining in medium intensity, 3 = positive staining in the majority of high-power fields, and 4 = strongly positive staining or clusters in the majority of high-power fields. Unburned skin was also evaluated for comparison.

Monoclonal antibodies. Following burn injury the animals received either saline or one of two mAb by intravenous injection, anti-L-selectin (CD62L) (Dreg 200) or anti-CD18 (R15.7), in a blinded fashion. Each of these mAb was a kind gift from Drs. Robert Rothlein and Kai Kishimoto (Boehringer Ingelheim Pharmaceuticals, Inc.).

Dreg 200 is a murine IgG directed against human L-selectin. This Ab cross-reacts with rabbits, and the IgG or Fab fragments inhibit leukocyte rolling in mesenteric postcapillary venules of rabbits [25].

R15.7 is a murine IgG2a which recognizes an epitope on the b chain of CD18 [19]. R15.7 has been demonstrated both *in vitro* and *in vivo* to block neutrophil adherence and migration to a variety of stimuli including LPS, phorbol myristate acetate, *N*-formylmethionylleucylphenylalanine, complement fragment, interleukin-1, and tumor necrosis factor. This mAb blocks CD18-mediated adhesion and inflammation in rabbits [19, 30].

R7.1 is a murine IgG2a directed to the a chain of human CD11a/CD18 (LFA-1) that inhibits firm adherence to ICAM-1, and is active in rabbits. R7.1 was used as a leukocyte marker for immunohistochemical study and was not administered *in vivo*.

Immunohistochemistry. Frozen skin sections, 8 μm thick, were cut at −15 to −30°C using a cryostat microtome (AO Reichert Scientific, Buffalo, NY), then normal skin and partial thickness burns from the same animal were placed on the same glass slide to ensure uniformity in staining technique. Fixed sections were stained with monoclonal antibodies against leukocyte CD11a using the biotin–streptavidin immunoperoxidase technique. Briefly, the primary antibody is applied to cut sections and a biotin-labeled secondary goat anti-murine Ig is used to identify the primary antibody. Peroxidase-labeled streptavidin is then added and the substrate, 3,3'-diaminobenzidine, is used to develop the brownish color which is detected under light microscopy. All reagents except the primary antibodies are obtained from Biogenex (San Ramon, CA). Tissue sections are then counterstained with hematoxylin (Immunon, Pittsburgh, PA), air dried, and preserved with a coverslip and GelTol aqueous mounting medium (Immunon).

Statistical analysis of the data was performed with analysis of variance and the Mann–Whitney U test and χ^2. Significance was assigned to P values <0.05. Values in text are given as means ± SD, figures represent means ± SEM.

RESULTS

There was no significant difference in baseline weight, hematocrit, or leukocyte counts among the three groups. Also these animals did not demonstrate any changes in body weight, hematocrit, heart rate, or fluid intake for the duration of the study. Study mortality was 0 and none of the animals developed elevated body temperature, leukocytosis, leukopenia, or wound suppuration. The total percentage of body surface area burn to each animal was <5% and did not result in detectable changes in behavior or feeding. There were no significant changes in cutaneous blood flow at shaved unburned skin sites in any of the experimental groups. Hematocrit remained unchanged in each group throughout the length of the study.

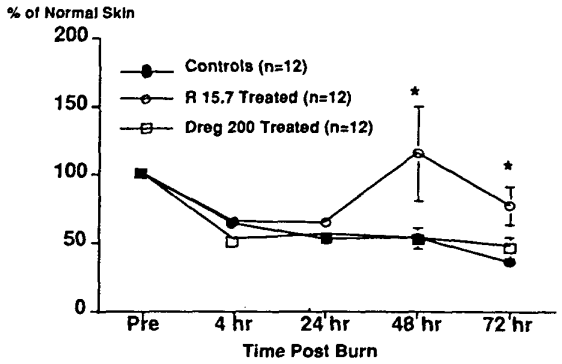

FIG. 1. Postburn skin blood flow (perfusion units). Relative changes in cutaneous blood flow in the zones of stasis determined by laser Doppler flowmeter are presented as percentage of baseline values (means ± SEM) for control animals ($n = 12$), animals given monoclonal antibody to CD18 (R15.7) ($n = 12$), and animals given the anti-L-selectin antibody (Dreg 200) ($n = 12$). Animals administered R15.7 had significantly greater blood flow in the zone of stasis at 48 and 72 hr than the controls or the Dreg 200 group (*$P < 0.05$).

To account for intergroup variations in baseline cutaneous blood flow, serial changes are compared as percentages of blood flow in unburned skin. Blood flow in burn contact sites demonstrated immediate and persistent decreases in perfusion to less than 20% of baseline blood flow which persisted through Day 3 postburn for all experimental groups. The consistent reduction in blood flow at the burn contact sites observed among all groups indicates the equivalence of the burn injury produced in each set of animals. On gross and histologic examination the burn contact sites of all groups were full-thickness injuries.

Serial blood flow measurements in the marginal zones of stasis are shown in Fig. 1. The control animals developed decrease in blood flow in the initial postburn period which was progressive through 72 hr to less than 40% of baseline. The R15.7 group had an initial decrease in blood flow at 4 hr and 24 hr postburn, similar to the controls, but recovered by 48 and 72 hr. By comparison the control group and the Dreg 200 group had persistent decreases in blood flow through the 72-hr period of observation.

The result of gross and histologic examination of the marginal zones of stasis are presented in Table 1. There was evidence of wound progression in the zone of stasis at 24 hr postburn, some of which progressed to full-thickness wounds at 72 hr. At 24 hr postburn, 7 of 12 zones in the control group showed evidence of progression, compared to 4 of 12 in the Dreg 200 group and 0

in those animals that received R15.7 ($P < 0.05$). After 72 hr, 9 of 12 control animals had full-thickness necrosis of the zones of stasis compared to 6 of 12 zones in the Dreg 200 animals and 1 of 12 animals in the R15.7 group.

Immunohistochemistry

There was no significant tissue staining for CD11a in unburned skin from animals in any of the experimental groups. The control animals and the R15.7 animals had significant leukocyte accumulation in the zone of stasis at 24 hr postburn, evidenced by intense positive staining (grade 3 and 4) in all control tissue samples and 11 of 12 samples in the R15.7 group. Leukocyte accumulation was somewhat less in the Dreg 200 group (see Fig. 2).

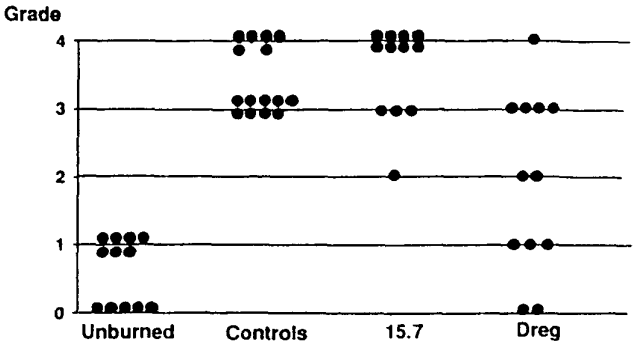

FIG. 2. 24-hr leukocyte accumulation in the zone of stasis for all groups of animals. Unburned skin did not have significant leukocyte accumulation. Leukocyte accumulation was higher in the control and R15.7 groups compared to unburned skin ($P < 0.05$). Differences between the Dreg and the control groups were not statistically significant.

DISCUSSION

The microvascular endothelium has become a focal point in understanding acute injury processes. Endothelial injury by PMNs is thought to occur as a result of the release of proteases and toxic oxygen products capable of injuring the endothelium and surrounding matrix and leading to increased endothelial permeability. When these substances are released in the circulation, they are inactivated by circulating antiproteases and free radical scavengers. The microenvironment formed between the endothelial cell and the PMN where the PMN-derived proteases and toxic oxygen products are present in high relative concentrations can act synergistically on the endothelial surface to cause injury. Endothelial injury involves formation of intercellular gaps, increased permeability, and PMN emigration into the extravascular matrix.

We have demonstrated that inhibition of leukocyte adherence with the anti-CD18 antibody R15.7 following burn injury improves microvascular perfusion in the marginal zone of stasis following thermal injury and prevents burn wound extension/progression when administered 30 min postinjury. These observations indicate that leukocytes are involved in the pathogenesis of local microvascular injury and extension of burn size in the zone of stasis, and suggest that these events can be modulated in the early phase following burn injury. The early reduction of cutaneous blood flow in animals given R15.7 is consistent with the observation of increased leukocyte accumulation at 24 hr postburn in this group of animals and suggests that early changes in the blood flow are unrelated to the presence of leukocytes and probably dependent on local hemodynamic factors such as vasoconstriction and activation of other inflammatory mediators. The late improvements in blood flow appear to be CD18 mediated; however, differences in leukocyte accumulation at 48 to 72 hr would be difficult to discern as most of these wounds show an intense leukocyte response.

Inhibition of L-selectin did not significantly alter blood flow or burn wound progression but was associated with reduced leukocyte accumulation at 24 hr. This observation is consistent with the role of selectins as mediators of leukocyte rolling and transient PMN–EC interactions as is the failure of Dreg 200 to completely prevent leukocyte accumulation. Other possibilities to explain the lack of efficacy of anti-L-selectin include (1) selectins are involved in transient PMN–EC interactions which are dependent on microvascular flow velocities, (2) other selectins such as E-selectin on endothelial cells may play a more dominant role in selectin-mediated adhesion, and (3) early changes in blood flow may be dependent on nonleukocyte mechanisms such as the kinin cascade, and leukocytes may be involved in a secondary injury, which would be consistent with the observations in the local tissue injury described above.

We conclude that progression of burn injury is related to late reduction in local skin perfusion which appears to be CD18 dependent. Early leukocyte accumulation in burn wounds is not associated with progression of burn wound necrosis.

TABLE 1

Progression of Burn Wound Necrosis

	24 hr	72 hr
Saline	7/12	9/12
R15.7	0/12*	1/12*
Dreg	4/12	6/12

* $P < 0.05$.

REFERENCES

1. Boykin, J. V., Eriksson, E., and Pittman, R. N. In-vivo microcirculation of a scald burn and the progression of post-burn dermal ischemia. *Plast. Reconstr. Surg.* **66**(2): 191, 1980.
2. Regas, F., and Ehrlich, H. Elucidating the vascular response to burns with a new rat model. *J. Trauma* **32:** 557, 1992.
3. Ward, P., and Till, G. Pathophysiologic events related to thermal injury of skin. *J. Trauma* **30:** s75, 1990.

4. Solomkin, J. S. Neutrophil disorders in burn injury: Complement, cytokines and organ injury. *J. Trauma* **30:** s80, 1990.
5. Herndon, D., and Traber, D. Pulmonary circulation and burns and trauma. *J. Trauma* **30:** s41, 1990.
6. Deitch, E. A. The management of burns. *N. Engl. J. Med.* **323:** 1249, 1990.
7. Harlan, J. M. Neutrophil mediated vascular injury. *Acta Chir. Scand. Suppl.* **715:** 123, 1987.
8. Movat, H. Z., and Cybulsky, M. I. Neutrophil emigration and microvascular injury. *Pathol. Immunopathol. Res.* **6:** 153, 1987.
9. Weiss, S. J. Tissue destruction by neutrophils. *N. Engl. J. Med.* **320:** 365, 1989.
10. Sacks, T., Moldow, C. F., Craddock, P. R., *et al.* Oxygen radicals mediate endothelial damage by complement stimulated granulocytes. *J. Clin. Invest.* **61:** 1161, 1979.
11. Shasby, D. M., Shasby, S. S., and Peach, M. J. Granulocytes and phorbol myristate acetate increase permeability to albumin of cultured endothelial monolayers and isolated perfused lungs. Role of oxygen radicals and granulocyte adherence. *Am. Rev. Respir. Dis.* **127:** 72, 1983.
12. Lasky, L. A. Selectins: Interpreters of cell-specific carbohydrate information during inflammation. *Science* **258:** 964, 1992.
13. Kishimoto, T. K., Warnock, M., Jutilla, M., *et al.* Antibodies against human LECAM-1 and ELAM-1 inhibit a common CD18-independent adhesion pathway in vitro. *Blood* **78:** 805, 1991.
14. Picker, L., Warnock, R., Burns, A., Doerschuck, C., Berg, E., and Butcher, E. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* **66:** 921, 1991.
15. Lawrence, M., and Springer, T. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell* **65:** 859, 1991.
16. Simon, S., Chambers, J., Butcher, E., and Sklar, L. Neutrophil aggregation is integrin and selectin dependent in blood and isolated cells. *J. Immunol.* **149:** 2765, 1992.
17. Price, T., Beatty, P. G., and Corpuz, S. R. In vivo inhibition of neutrophil function in the rabbit using monoclonal antibody to CD18. *J. Immunol.* **139:** 4174, 1987.
18. Barton, R. W., Rothlein, R., Ksiazek, J., and Kennedy, C. The effect of anti-intercellular adhesion molecule-1 on phorbol-ester-induced rabbit lung inflammation. *J. Immunol.* **143:** 1278, 1989.
19. Argenbright, L., Letts, L., and Rothlein, R. Monoclonal antibodies to the leukocyte membrane CD18 glycoprotein complex and to intercellular adhesion molecule-1 inhibit leukocyte-endothelial adhesion in rabbits. *J. Leukocyte Biol.* **49:** 253, 1991.
20. Walsh, C., Carey, D., Cok, D., Bechard, C., Fowler, A., and Sugarman, H. Anti-CD18 antibody attenuates neutropenia and alveolar capillary-membrane injury during gram-negative sepsis. *Surgery* **110:** 205, 1991.
21. Simpson, P., Todd, R., Fantone, J., *et al.* Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody that inhibits leukocyte adhesion. *J. Clin. Invest.* **81:** 624, 1988.
22. Mileski, W. J., Borgstrom, D., Lightfoot, E., Rothlein, R., Faanes, R., Lipsky, P., and Baxter, C. Inhibition of leukocyte-endothelial adherence following thermal injury. *J. Surg. Res.* **52:** 334, 1992.
23. Mileski, W., Raymond, J., Winn, R., Harlan, J., and Rice, C. Inhibition of leukocyte adherence and aggregation for treatment of severe cold injury in rabbits. *J. Appl. Physiol.* **74:** 1432, 1993.
24. Ngim, R. C. The burned ear. I. An experimental study with the rabbit model to evaluate scalding temperature, surface and histopathologic appearance, and healing responses with depth of injury. *Ann. Acad. Med. Singapore* **21:** 597, 1992.
25. Stern, M. D. In vivo evaluation of microcirculation by coherent light scattering. *Nature* **254:** 56, 1975.
26. Stern, M. D., Lappe, D. L., Bowen, P. D., *et al.* Continuous measurement of tissue blood flow by laser–Doppler spectroscopy. *Am. J. Physiol.* **232**(4): H441, 1977.
27. Nilsson, G. E., Tenland, T., and Oberg, P. A. Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow. *IEEE Trans. Biomed. Eng.* **27**(10): 597, 1980.
28. Alsbjorn, B., Micheels, J., and Sorensen, B. Laser Doppler flowmetry measurements of superficial, dermal, deep dermal and subdermal burns. *Scand. J. Plast. Reconstr. Surg.* **18:** 75, 1984.
29. Green, M., Holloway, A., and Heimbach, D. Laser Doppler monitoring of microcirculatory changes in acute burn wounds. *J. Burn Care Rehabil.* **9:** 57, 1988.
30. Simon, S. I., Chambers, J. D., Butcher, E., and Sklar, L. A. Neutrophil aggregation is β2-integrin- and L-selectin-dependent in blood and isolated cells. *J. Immunol.* **149:** 2765, 1992.

JOURNAL OF SURGICAL RESEARCH

CLINICAL AND LABORATORY INVESTIGATION

Official Organ of the Association for Academic Surgery
Official Organ of the Association of Veterans Administration Surgeons

Volume 63, Number 1, June 1996

JOURNAL OF SURGICAL RESEARCH

Volume 63, Number 1, June 1996

http://www.apnet.com

0022-4804/96 $18.00

MADE IN THE UNITED STATES OF AMERICA

This journal is printed on acid-free paper.

♾

JOURNAL OF SURGICAL RESEARCH

(ISSN 0022-4804)

Published monthly (except semimonthly in February and July) by Academic Press, Inc.,
6277 Sea Harbor Drive, Orlando, FL 32887-4900

1996: Volumes 60–66. Price: $598.00 U.S.A. and Canada; $726.00 all other countries
All prices include postage and handling.

Information concerning personal subscription rates may be obtained by writing to the Publishers. All correspondence and subscription orders should be addressed to the office of the Publishers at 6277 Sea Harbor Drive, Orlando, FL 32887-4900. Send notices of change of address to the office of the Publishers at least 6 to 8 weeks in advance. Please include both old and new addresses. POSTMASTER: Send changes of address to *Journal of Surgical Research*, 6277 Sea Harbor Drive, Orlando, FL 32887-4900.

Periodicals postage paid at Orlando, FL, and at additional mailing offices.